STUDIES OF THE SOLUBLE PHOSPHOPROTEINS OF LIVER AND THE PURIFICATION AND PROPERTIES OF A PHOSPHATASE FROM SPLEEN

By

JOHN GLOMSET

UPPSALA 1960

ALMQVIST & WIKSELLS BOKTRYCKERI AB

STUDIES OF THE SOLUBLE PHOSPHOPROTEINS OF LIVER AND THE PURIFICATION AND PROPERTIES OF A PHOSPHATASE FROM SPLEEN

By

JOHN GLOMSET
Med. kand., G.-H.

AKADEMISK AVHANDLING

som med vederbörligt tillstånd
av Kanslern för Rikets Universitet för vinnande av
medicine doktorsgrad framlägges till offentlig granskning å
Medicinsk-Kemiska Institutionens föreläsningssal
lördagen den 6 februari 1960
kl. 10 fm.

UPPSALA 1960
ALMQVIST & WIKSELLS BOKTRYCKERI AB

From the Institute of Medical Chemistry, University of Uppsala, Uppsala, Sweden.

Studies of the Soluble Phosphoproteins of Liver and the Purification and Properties of a Phosphatase from Spleen

by

JOHN A. GLOMSET

Prefatory Note

This paper is a summary of the following communications which will be referred to by Roman numerals as designated below:

I. J. Glomset, Fractionation of the Phosphorus-Containing Proteins of Rat Liver Cell Supernatant. Acta Chem. Scand. 11, 512 (1957).

II. J. Glomset, The Fractionation of Rat Liver Supernatant by Salt Gradient Elution from Triethylaminoethyl Cellulose Columns. Acta Chem. Scand. 12, 641 (1958).

III. J. Glomset, The Incorporation In Vitro of Radioactive Phosphorus into the Proteins of Rat Liver Supernatant. Acta Soc. Med. Upsaliensis 64, 236 (1959).

IV. J. Glomset, The Further Purification and Properties of a Phosphatase from Spleen Able to Hydrolyze Completely the Phosphorus of α-Casein. Biochim. Biophys. Acta 32, 349 (1959).

V. J. Glomset and J. Porath, Some Properties of a Phosphatase from Bovine Spleen. Biochim. Biophys. Acta (In Press).

VI. J. Glomset, The Incorporation *In Vitro* of Inorganic Phosphate into a Soluble Protein of Rat Liver. Acta Soc. Med. Upsaliensis (In Press).

Introduction

The first indication of the occurrence of intracellular phosphoproteins derived from the work of Plimmer and Scott in 1908 (1). These authors

extended the observation (2) that the phosphorus of casein is alkali-labile, and showed that this type of phosphorus was present in cells. Almost 40 years later, in 1945, these findings were confirmed and incorporated into a general method for the fractionation of cellular phosphorus compounds by Schmidt and Thannhauser (3). In this method the low-molecular-weight, water-soluble phosphorus compounds are extracted from cells with cold TCA.[1] Lipid phosphorus is extracted as a separate fraction using organic solvents. Finally, RNA, phosphoprotein, and DNA phosphorus are separated by virtue of the different behavior of these classes of compounds when subjected to the action of 1 *N* alkali at 37°C. Thus, the phosphorus of RNA, while still bound in organic linkage becomes acid soluble. In contrast the so-called phosphoprotein phosphorus is liberated as inorganic phosphate—by definition, in analogy with casein. Finally, the DNA phosphorus remains acid insoluble. The method of Schmidt and Thannhauser is very useful for obtaining a preliminary estimate of the relative amounts of phosphorus compounds in cells, but suffers from the disadvantage that the proteins are denatured during the course of the procedure and that the nature of the protein-phosphorus bond is of necessity less amenable to study. A second method, also published in 1945, viz. that of Schneider (4), also suffers from the first-mentioned difficulty. However, the method of Schneider has an advantage over that of Schmidt and Thannhauser with respect to phosphoprotein phosphorus because it yields an essentially nucleic acid-free protein fraction containing organically bound phosphorus. Thus, the nature of the phosphorus linkage may be studied more closely. Proceeding from the protein fraction obtained by this technique, it was possible in this laboratory to isolate phosphoserine and phosphothreonine from partial acid hydrolysates of cellular proteins, not only from liver and other mammalian organs (5, 6), but also from bacteria (7) and yeast (8). Moreover, it was possible to show that the phosphoserine of cellular phosphoproteins was active metabolically as judged from the rapidity with which radioactive phosphate was incorporated into the proteins *in vivo* (6, 9). This supported the hypothesis that the phospho-

---

[1] The following abbreviations will be employed:

TCA = trichloroacetic acid
RNA = ribonucleic acid
DNA = desoxyribonucleic acid
^{32}P = radioactive phosphate
iP = inorganic phosphate
ATP = adenosinetriphosphate
SerP = phosphorylserine
ThrP = phosphoryl threonine
Tris = tris(hydroxymethyl)aminomethane
TEAE = triethylaminoethyl cellulose

proteins of cells were not, for example, inert structural elements but were actively taking part in intracellular metabolic processes. However, it was clear that further evidence would have to derive from work with native, phosphorus-containing proteins in cell-free systems. Important papers were soon forthcoming. Burnett and Kennedy found an enzyme in rat liver mitochondria which can catalyze the incorporation of radioactive phosphate from $AT^{32}P$ into unlabelled casein *in vitro* (10). And Sutherland and coworkers found that the phosphoprotein, liver phosphorylase could be inactivated by a specific phosphatase and reactivated by a specific phosphokinase (11, 12). Thus, the results of both of these groups provided mechanisms for the incorporation of phosphorus into intracellular proteins, in both cases ultimately dependent on the presence of ATP. Also, in preliminary experiments Ågren and Engström found that a partially purified hexokinase preparation from yeast was able to incorporate ^{32}P into SerP of the protein in the presence of $AT^{32}P$ (13). For a recent, more complete review of these and other investigations concerning the chemistry and metabolism of intracellular phosphoproteins, see Ågren's monograph (14).

The present investigation was performed in continuation of the previous studies (5, 6). Thus, it had been found (6) that relatively soon following the injection of $i^{32}P$ into rats the isotope could be demonstrated in the phosphoserine of liver, kidney, heart, spleen, intestinal mucosa, and brain. Moreover, the phosphoproteins of liver were found to be of special interest, since of the above-mentioned organs the liver showed the most extensive uptake of the isotope into the protein SerP. Further investigation of the SerP-protein of rat liver revealed that each of the subcellular fractions obtained by the differential centrifugation technique of Schneider and Hogeboom (15) contained the protein-bound, phosphorylated amino acid (6).

Since unpublished observations by the author had indicated that of the various subcellular fractions the "supernatant" contained the highest $Ser^{32}P$ activity 1 hour after injection of $i^{32}P$, this fraction was selected for further study. Since phosphorylase is mainly located in the supernatant (16), it was presumed that some of the $Ser^{32}P$ of the supernatant probably derived from that phosphoprotein enzyme. However, it was also considered likely that other proteins might be present which could also incorporate the isotope, and there was a distinct possibility that these could be demonstrated using some of the more recent techniques of protein fractionation. The plan therefore adopted for the investigation was to attempt to develop suitable methods for the fractionation of the "supernatant" proteins of rat liver with particular emphasis on the phosphorus-containing proteins, and then to employ these methods in studying the *in vivo* and if possible the *in vitro* incorporation of

radioactive phosphate into the protein fractions. The present paper comprises a summary of the work which has so far been performed, the several sections corresponding to the separate publications (I–VI) listed in the preface.

I. Fractionation of the Phosphorus-Containing Proteins of Rat Liver Cell Supernatant

In these studies general methods were sought which would make possible a simple, reproducible fractionation of the soluble proteins of rat liver. In doing so a number of considerations had to be taken into account. In-bred, adult male Wistar rats from the departmental colony were used in all experiments. Particular care was taken to perfuse the rat livers as quickly as possible, at the same time reducing the temperature of the liver with ice-cold sucrose solution. The interval elapsing between the time when a given animal was killed by cervical dislocation until its liver, chilled and perfused was immersed in an ice-cold sucrose bath usually did not exceed three minutes. Immediately after obtaining the "supernatant" fraction from the homogenized liver by differential centrifugation (15), fractionation of the proteins was begun. Two different techniques were found to be particularly suitable for this purpose. The first was zone electrophoresis on columns of ethanolized cellulose (17) after a preliminary ammonium sulfate fractionation. The second was stepwise elution chromatography from TEAE-Cl columns.

With respect to the first procedure, it was found that the direct precipitation of the supernatant proteins at 50% and 85% ammonium sulfate saturation had several advantages. It accomplished a reduction in volume of the sucrose supernatant, the removal of lower molecular weight compounds, and a rather sharp separation of the proteins into two fractions with distinctive electrophoretic properties. This facilitated the subsequent zone electrophoresis, permitting a final separation into several different protein peaks.

In the experiments with the cellulose ion exchange columns no preliminary ammonium sulfate fractionation was necessary. However, it was found that a significant amount of the total soluble liver phosphoprotein was so strongly adsorbed by the column that it was removable only with 1% NaOH. Nevertheless, a clear-cut separation of the remaining proteins was obtained, as judged from the subsequent electropherograms.

In the case of both the electrophoretic and the chromatographic procedures, it was found that Tris-HCl buffers could be used to good advantage, thus circumventing the use of phosphate buffers in the fractionation experiments. Although the latter have frequently been used with success in the case of

other proteins, it seemed inadvisable to employ phosphate buffers with phosphoproteins because of the possibility of exchange and adsorption.

When the above methods were employed for the fractionation of supernatant phosphoproteins, which had been labelled by the intraperitoneal injection of $i^{32}P$ into rats one hour before the start of the experiment, it was possible to isolate $Ser^{32}P$ in significant amounts from three of the supernatant subfractions. Two of these were present in the 50% saturated ammonium sulfate precipitate and third in the 85% precipitate.

II. The Fractionation of Rat Liver Supernatant by Salt Gradient Elution from Triethylaminoethyl Cellulose Columns

In continued methodological experiments the fractionation of rat liver supernatant on TEAE-Cl columns was studied using chloride gradients at pH 7.4. A two stage gradient system was employed: from 0.01 *M* Tris-HCl, pH 7.4 to 0.01 *M* Tris-HCl + 0.2 *M* NaCl, and finally from 0.01 *M* Tris-HCl + 0.2 *M* NaCl to 0.01 *M* Tris-HCl + 1 *M* NaCl. Using this system a separation of nucleotides, proteins, and nucleotide-protein mixtures could be achieved. The separation of nucleotide-protein mixtures using the same chromatographic system was of particular interest. Thus, it opened up the possibility that after incubation of the supernatant proteins *in vitro* with, for example, radioactive inorganic phosphate or adenosine triphosphate, proteins which had become labelled could subsequently be separated from the radioactive lower molecular weight compounds in a single simple step. In cases where this type of separation could be achieved, the study of the *in vitro* incorporation of phosphate—as well as other radicals or compounds—into proteins would be considerably facilitated.

III. The Incorporation *in vitro* of Radioactive Phosphorus into the Proteins of Rat Liver Supernatant

Having shown (I and II) that both zone electrophoresis and TEAE chromatography could be used in fractionation of the soluble phosphoproteins of rat liver and that these proteins could be labelled with radioactive phosphorus not only *in vivo* but also *in vitro* (preliminary experiments in I), further attempts were made to study the *in vitro* incorporation in more detail. It was found that there were at least two different types of incorporation reaction. One type was dependent on ATP and Mg ions. The second required only iP, the addition of external ions being unnecessary. The ATP-dependent

incorporation occurred in the protein fraction precipitating at 50% ammonium sulfate saturation. Moreover, using the zone electrophoresis procedure described previously (I) evidence was obtained suggesting that two of the electrophoretic subfractions were active. In contrast to the ATP-dependent, ^{32}P-incorporating proteins, the i^{32}P-incorporating fraction was located in the supernatant protein fraction precipitating at 85% ammonium sulfate saturation. In addition, the active, i^{32}P-incorporating protein could be further fractionated using the gradient elution procedure on TEAE-Cl columns described earlier (II). With this technique a considerable purification was accomplished; moreover, after incubation of the protein with i^{32}P it was possible to separate the ^{32}P-protein from excess i^{32}P.

Thus, the combination of methods developed in I and II was successfully employed in III. Furthermore, the partial loss of phosphoprotein found to occur when the supernatant was chromatographed directly on TEAE-Cl could be circumvented by preliminary ammonium sulfate precipitation of the soluble proteins and the subsequent use of zone electrophoresis and TEAE-Cl chromatography for the fractions precipitating at 50% and 85% ammonium sulfate saturation, respectively.

The identity of the respective ^{32}P-incorporating proteins has not yet been determined. With respect to the proteins exhibiting AT^{32}P-dependent P incorporation, it is known that liver phosphorylase is present in the precipitate obtained at 50% ammonium sulfate saturation and that this protein is able to incorporate ^{32}P into SerP *in vitro* (12, 18) under conditions similar to those employed here. The identity of the second phosphoprotein in the 50% ammonium sulfate fraction, however, remains to be established. Moreover, this question is of considerable metabolic interest in view of the possibility that hormonal regulation of a phosphorylation-dephosphorylation cycle might exist similar to that elucidated by Sutherland and coworkers in their excellent studies of liver phosphorylase (11, 12).

IV. The Further Purification and Properties of a Phosphatase from Spleen Able to Hydrolyze Completely the Phosphorus of α-Casein

Although both zone electrophoresis and ion exchange chromatography had been found useful for work with phosphoproteins, it was recognized that other types of methods for the study of phosphoproteins were also necessary. For example, if a protein fraction, known to contain a given enzyme, were shown to contain or incorporate phosphorus, one way of showing that the

phosphorus was actually necessary for the activity of the enzyme would be to determine the effect of dephosphorylation on the enzyme activity. This type of experiment has been performed by Sutherland and coworkers using liver phosphorylase phosphatase (11, 12). They were able to show that partial dephosphorylation completely inhibited the activity of liver phosphorylase and that the activity was recovered on rephosphorylation.

In searching for a more general method for accomplishing the enzymic dephosphorylation of phosphoproteins, the so-called phosphoprotein phosphatase of bovine spleen was studied, primarily in order to more closely define its properties and if possible to obtain a purer preparation. This was particularly necessary if relatively pure, well-defined phosphoproteins were to be dephosphorylated. Thus, the ideal experiment would be to add pure phosphatase to a pure phosphoprotein, incubate for a suitable period, and then be able to separate and identify the respective proteins from the reaction mixture.

The existence of a specific phosphoprotein phosphatase was first suggested in 1946 by the work of Harris (19). Since then a number of enzymes have been found which dephosphorylate casein while having little or no action towards the commonly used phosphomonoester, β-glycerophosphate, thus differentiating them from other phosphatases such as prostatic acid phosphatase or intestinal alkaline phosphatase. Because of the properties of these enzymes mentioned above they have been called phosphoprotein phosphatases, it being assumed that they are also able to dephosphorylate phosphoproteins other than casein. This type of enzyme has been demonstrated in several mammalian organs, spleen having been found to contain particularly large quantities (20). Of the two methods then available for the preparation of the crude spleen enzyme (20, 21), that of Singer and Fruton was employed by the author as a suitable starting material for attempts to further purify and characterize the phosphatase. It was found that the latter could be purified considerably using a combination of TEAE chromatography and zone electrophoresis, yielding a basic, blue-violet protein which moved as a single peak on electrophoresis in NaAc-HAc buffer, pH 5.6. The purified enzyme was found to be able to completely dephosphorylate casein, *p*-nitrophenylphosphate and to hydrolyze the phosphate bonds of iPP, ATP, and phosphoamidate.

V. Some Properties of a Phosphatase from Bovine Spleen

In experiments designed to further characterize the spleen phosphatase several chemical and physical properties of the enzyme were measured. The enzyme appeared to be homogeneous in buffers of three different pHs, viz.

2.4, 6.0, and 8.1, in electrophoretic mobility experiments made in the moving boundary apparatus. In all cases the enzyme moved toward the cathode, indicating a probable high ionization point. Unfortunately, a more exact determination of the latter could not be made due to the irreversible denaturation of the enzyme occurring above pH 8. The sedimentation behavior of the enzyme could also be taken as an indication of homogeneity, only one sedimenting boundary being observed. Carbohydrate analysis of the purified protein revealed a hexosamine content of 1.45% and a hexose content of 3.21%. Calculations of molecular weight based on sedimentation data and carbohydrate content gave an approximate value of 23,000. Preliminary, unpublished experiments at the Institutes of Biochemistry in Uppsala and Medical Chemistry in Lund have indicated that the qualitative amino acid content of the protein is not remarkable but that alanine is the sole *N*-terminal amino acid. Optical measurements indicate the enzyme to have two absorption maxima in addition to the expected peak at 280 mμ, viz. a broad peak at 550 mμ and also a maximum at 320 mμ. Although the nature of the blue-violet chromophore is at present unknown, active attempts are now being made to more closely investigate the structure and mechanism of action of the enzyme.

VI. The Incorporation *in vitro* of Inorganic Phosphate into a Soluble Protein of Rat Liver

Having obtained an extremely pure preparation of bovine spleen, "phosphoprotein phosphatase", the enzyme was used in conjunction with a further study of the soluble i^{32}P-incorporating protein of rat liver. This particular protein was of especial interest, since the incorporation of free, inorganic phosphate does not usually occur in proteins. If, for example, 100 mg of "Hammarsten casein" are incubated in 0.01 *M* Tris-HCl, at pH 7.4 and 21°C with 1 mC i^{32}P for 15 min., no Ser^{32}P can be demonstrated in a partial acid hydrolysate of the protein. Nor does this type of reaction occur with any of the many other soluble proteins of rat liver. Yet, this is what happens in the case of the active liver protein.

It was considered of particular importance to attempt to determine the nature of some of the factors responsible for the incorporation reaction. Experiments were performed which suggest that a special configuration of the protein is required which is sensitive both to heat pretreatment and to the presence of urea. Furthermore, it was shown that the protein is sensitive to both parachloromercuribenzoate and iodoacetamide, indicating that a SH group or groups may be of importance in maintaining the i^{32}P-incorporating

activity of the protein. In experiments employing purified protein fractions the incorporation reaction was shown to be reversible, and it therefore seems probable that the reaction is an exchange reaction, dependent on a specific structure of the active protein.

It was originally planned to further study the conditions of the incorporation using active protein which had been dephosphorylated previously. If $i^{32}P$ could be shown to be incorporated into the dephosphorylated protein, something in addition to an exchange reaction would have to be postulated, e.g. a *de novo* phosphorylation of the protein. However, when the ^{32}P-protein was incubated with relatively large amounts of the purified "phosphoprotein phosphatase", no appreciable dephosphorylation could be demonstrated under the experimental conditions. In seeking an explanation for this lack of activity with respect to the ^{32}P-protein, it seems likely that the enzyme is specific for a certain type of amino acid configuration surrounding a given phosphorylated amino acid and that this configuration is absent in the present protein. Thus, it is known that in casein the SerP occurs in conjunction with large amounts of acidic amino acids (22) and recent evidence (23) indicates that diSerP peptides exist in casein. It may be that in the absence of surrounding acidic amino acids the enzyme has much less affinity for a phosphorus-containing protein or peptide. In the simplest case, viz. SerP itself, it is known that the purified enzyme has very little activity (unpublished experiments). The interesting problem of the specificity of the purified enzyme is now under active study in this laboratory. It is hoped that the information gained in this study will make the spleen phosphatase a still more important tool for the study of phosphoprotein structure and function.

The incorporation of $i^{32}P$ into the present liver protein may involve what might be called "active" serine. There are several known instances in which a particular serine residue of a protein appears to have special reactive properties. Thus, it has been known for several years that compounds such as diisopropylfluorophosphonate are able to inactive various esterases and proteolytic enzymes and that a specific serine residue of the protein molecule becomes phosphorylated in the process (24). Furthermore, Koshland and Erwin (25) have called attention to a very interesting parallel between these enzymes and muscle phosphoglucomutase. Exchange of the phosphorus of a specific serine residue of the latter enzyme occurs in the course of the phosphoglucomutase reaction. And Koshland and Erwin have shown (25) that the sequence of amino acids surrounding the "active" serine of this enzyme resembles that of the enzymes mentioned above. This suggests that the primary structure in the vicinity of the respective serine groups might be of particular importance in activating the serine.

It would therefore be of considerable interest to know whether a similar structure contributes to the particular reactivity of the serine of the present liver protein. Moreover, other instances of phosphorylated "active" serine are also known, notably in the case of the alkaline phosphatases of intestinal mucosa and bone (*26*, *27*). These might also fit into the same picture. In any event it is likely that the "activation" of serine is an important feature of the catalytic activity of many enzymes, and there is no question but that the mode of this "activation" is worthy of much further study.

Summary

Methods have been developed for the fractionation of the soluble proteins of liver, employing both zone electrophoresis and ion exchange chromatography. The soluble phosphoproteins have been the object of special study, in a continuation of work performed earlier in this laboratory. Both the *in vivo* and the *in vitro* incorporation of radioactive phosphate into the phosphoproteins has been followed. Two fractions have been found to incorporate radioactive phosphate in the presence of radioactive adenosinetriphosphate and Mg ions. In an additional fraction an exchange with inorganic phosphate of the medium has been demonstrated. The latter reaction has been studied in more detail in different types of experiments, among them dephosphorylation experiments using a phosphatase which has been obtained in what appears to be pure form from extracts of bovine spleen.

Acknowledgements

I am grateful to Professor Gunnar Ågren, who introduced me to the study of phosphoproteins, and in whose laboratory I have been privileged to carry out this work. I also wish to thank Dr. Jerker Porath for his unfailing interest, stimulating criticism, and for his collaboration in Paper V. My sincere thanks are also due to all of the members of the Institute of Medical Chemistry for countless services during the past years.

References

1. Plimmer, R. H. A., and Scott, F. H., *J. Chem. Soc.*, (London), *93*, 1699 (1908).
2. Plimmer, R. H. A., and Bayliss, W. M., *J. Physiol.*, *33*, 465 (1906).
3. Schmidt, G., and Thannhauser, J., *J. Biol. Chem.*, *161*, 83 (1945).
4. Schneider, W. C., *J. Biol. Chem.*, *161*, 293 (1945).

5. Ågren, G., de Verdier, C.-H., and Glomset, J., *Acta Chem. Scand., 8,* 503 (1954).
6. Ågren, G., de Verdier, C.-H., and Glomset, J., *Acta Chem. Scand., 8,* 1570 (1954).
7. Ågren, G., de Verdier, C.-H., and Glomset, J., *Acta Chem. Scand., 9,* 196 (1955).
8. Engström, L., and Ågren, G., *Acta Chem. Scand., 10,* 877 (1956).
9. Ågren, G., de Verdier, C.-H., and Glomset, J., *Revue de Ferment. et des Industr. Aliment., 11,* 234 (1956).
10. Burnett, G., and Kennedy, E. P., *J. Biol. Chem., 211,* 969 (1954).
11. Wosilait, W. O., and Sutherland, E. W., *J. Biol. Chem., 218,* 469 (1956).
12. Rall, T. W., Sutherland, E. W., and Wosilait, W. O., *J. Biol. Chem., 218,* 483 (1956).
13. Ågren, G., and Engström, L., *Acta Chem. Scand., 10,* 489 (1956).
14. Ågren, G., *Acta Univ. Upsaliensis, 5* (1958).
15. Schneider., W. C., and Hogeboom, G. H., *J. Biol. Chem., 183,* 123 (1950).
16. Hers, H. G., Berthet, I., Berthet, C., and de Duve, C., *Bull. Soc. Chim. Biol., 33,* 21 (1951).
17. Porath, J., *Biochim. et Biophys. Acta, 22,* 151 (1956).
18. Wosilait, W. O., *J. Biol. Chem., 233,* 597 (1958).
19. Harris, D. L., *J. Biol. Chem., 165,* 541 (1949).
20. Sundararajan, T. A., and Sarma, P. S., *Biochem. J., 56,* 125 (1954).
21. Singer, M. F., and Fruton, J. S., *J. Biol. Chem., 229,* 111 (1957).
22. Perlmann, G. E., *Advances in Protein Chem.,* Vol. 10, page 1 (1955).
23. Williams, J., and Sanger, F., *Biochem. et Biophys. Acta, 33,* 294 (1959).
24. Davies, D. R., and Green, A. L., *Advances in Enzymology,* Vol. 20, page 283 (1958).
25. Koshland, D. E. Jr., and Erwin, M. J., *J. Am. Chem. Soc., 79,* 2657 (1957).
26. Engström, L., and Ågren, G., *Acta Chem. Scand., 12,* 357 (1958).
27. Ågren, G., Zetterqvist, Ö., and Ojamäe, M., *Acta Chem. Scand., 13,* 1047 (1959).